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Angiotensin II-mediated signal transduction events in cystic fibrosis pancreatic duct cells

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Abstract

Different signal transduction pathways, i.e. Ca^{2+}- and cAMP-dependent, involved in mediating the effects of angiotensin II (AII) were investigated separately using the short-circuit current (I_{sc}) technique and radioimmunoassay (RIA) in a cystic fibrosis pancreatic cell line (CFPAC-1) which exhibits defective cAMP-dependent but intact Ca^{2+}-dependent anion secretion. The AII-induced I_{sc} could be inhibited by the specific antagonist for AT_1, losartan (1 μM), but not the antagonist for AT_2, PD123177 (up to 10 μM). The AII-induced I_{sc} was also reduced by the treatment of the cells with a Ca^{2+} chelator, BAPTA-AM (100 μM), indicating a dependence of the AII-induced anion secretion on the intracellular Ca^{2+}. Treatment of the cells with pertussis toxin (0.1 μg/ml) or a phospholipase C (PLC) inhibitor, U73122 (5 μM), resulted in a substantial reduction in the AII-induced I_{sc} indicating involvement of Gi and PLC in the Ca^{2+}-dependent anion secretion. RIA measurements showed that AII stimulated an increase in cAMP production which could be reduced by losartan, pertussis toxin and U73122 but not BAPTA-AM. In addition, inhibitors of cyclooxygenase, indomethacin (10 μM) and piroxicam (10 μM), did not have any effect on the AII-induced cAMP production, excluding the involvement of prostaglandins. Our results suggest that both AII-stimulated cAMP and Ca^{2+}-dependent responses are mediated by the AT_1 receptor and Gi-coupled PLC pathway. However, the AII-stimulated cAMP production in CFPAC-1 cells is not dependent on Ca^{2+} or the formation of prostaglandins.

Keywords: Angiotensin II; AT_1 receptor; cAMP; Ca^{2+}; CFPAC-1 cell

1. Introduction

Angiotensin II (AII) is known to act as an active physiologic regulator in the brain, kidney, vascular smooth muscle and adrenal gland (for review see [1]). A variety of signal transduction pathways can be elicited by AII, which include phospholipase C (PLC), phospholipase A_2 (PLA_2), adenylate cyclase and direct coupling of ion channels (for reviews see [2,3]). While AII-induced PLC-linked Ca^{2+} mobilization has been shown to be the dominant pathway in many target tissues [4,5], the signaling mechanism leading to AII-induced changes in cAMP levels is controversial. It has been shown that AII induces a decrease in cAMP production by the action of AT_1 receptor coupled to Gi exerting an inhibitory effect on adenylate cyclase in many tissues including the liver, pituitary gland, kidney, aorta and testis [3]. In contrast to the inhibition of adenylate cyclase by AII in other tissues, AII has been shown to increase cAMP levels in fetal skin fibroblasts [6,7]. The sig-

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naling mechanism involved in mediating the effect of AII on cAMP production in these cells is not clear.

A stimulatory effect of AII on Cl^- secretion has been noted in a number of epithelia including the airway [8,9], intestine [10] and epididymis [11]. However, the signal transduction events involved in mediating the AII responses remain largely unknown. Although an increase in cAMP production via prostaglandin formation has been implicated in mediating the AII-induced secretory responses, a Ca^{2+}-dependence of the responses to AII has also been observed [8,12].

Most Cl^--secreting epithelia possess both cAMP- and Ca^{2+}-dependent regulatory pathways [13–16], which may sometimes cross-talk with one another [17,18]. Both increases in cAMP and Ca^{2+} give rise to secretory responses in many epithelial cells and, therefore, this posts a difficulty in delineating the signal transduction events underlying AII-induced secretory responses. A cystic fibrosis pancreatic duct cell line, CFPAC-1, offers an advantage in this regard in that it exhibits intact Ca^{2+}-dependent but defective cAMP-dependent anion secretion [19]. Therefore, the AII-stimulated Ca^{2+}-dependent pathway can be studied using the short-circuit current (I_{sc}) technique which measures the secretory response. On the other hand, the AII-stimulated cAMP production can be measured by radioimmunoassay (RIA). The present study has made the attempt to elucidate the AII-mediated signal transduction events using the CF cell line in conjunction with these techniques.

2. Materials and methods

2.1. Materials

Iscove's modified Dulbecco's medium, Hanks' balanced salt solution (HBSS), trypsin, angiotensin II (AII), pertussis toxin (PTX) and Dulbecco's phosphate buffer saline (DPBS) were purchased from Sigma (St. Louis, MO). Fetal bovine serum was from Gibco Laboratories (New York). Sylgard resin (184 silicone slastemer kit) and silicone rubber (3140 RTV), for making the permeable supports, were purchased from Dow Corning (Midland, MI). 0.45 μm Millipore filters were obtained from Millipore (Bedford, MA). BAPTA-AM and U73122 were purchased from Calbiochem (La Jolla, CA). Losartan and PD123177 were a generous gift from the Du Pont Merck Pharmaceutical Company.

2.2. Cell culture

The culture procedure for CFPAC-1 cells was similar to that described previously [20]. The cells were grown in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum. The cells (1.2×10^6 cells/ml) were plated onto each floating permeable support which was made of Millipore filter with a silicone rubber ring attached on top of it for confining the cells. Before the cells were plated, the permeable supports were placed carefully on to culture medium so that they could float on top of the surface. Cultures were incubated at 37°C in 5% CO_2/95% air atmosphere, and reached confluence in 3–4 days. Variations between batches of cultured cells did exist. This was taken into consideration by performing independent control experiments for each batch of cultured cells

2.3. Short circuit current measurement

The measurement of I_{sc} has been described previously [20,21]. Monolayers grown on permeable supports were clamped vertically between two halves of the Ussing chamber. Monolayers were bathed in both sides with Krebs-Henseleit (K-H) solution which was maintained at 37°C by a water jacket enclosing the reservoir. The K-H solution had the following composition (mM): NaCl, 117; KCl, 4.5; $CaCl_2$, 2.5; $MgSO_4$, 1.2; $NaHCO_3$, 24.8; KH_2PO_4, 1.2; glucose, 11.1. The solution was bubbled with 95% O_2 and 5% CO_2 such that the pH of the solution was maintained at 7.4. Drugs could be added directly to the apical or basolateral side of the epithelium. Usually, the epithelium exhibited a basal transepithelial PD which was measured by the Ag/AgCl reference electrodes (Metrohm, Switzerland) connected to a preamplifier that was in turn connected to a voltage-clamp amplifier (World Precision Instruction, DVC-1000).

2.4. Measurement of cellular cAMP content

Cells (4 days) grown on 24-well plates were washed twice with physiological salt solution and incubated at 32°C for 10 min in the same buffer (0.5 ml/well) containing IBMX (3 mM, to inhibit hydrolysis of cAMP) either under control or other experimental conditions specified in Section 3. Concentrated perchloric acid (10 μl) was then added to each well to arrest cellular metabolism and extract cAMP; these extracts were neutralized with KOH and stored at −18°C until their cAMP contents were determined by radioimmunoassay (Amersham kit). The unit was given in fmol/10^7 cells. An AII concentration of 100 μM, which was much higher than that used to elicit I_{sc}, was used to elicit cAMP production, probably due to the differences in the sensitivities of the two techniques or in the two culture conditions affecting levels of receptor expression.

2.5. Statistical analysis

Results are expressed as mean ± standard error of the mean (S.E.M.). Comparisons between groups of data were made by Student's unpaired *t*-test. A *P* value of less than 0.05 was considered statistically significant.

3. Results

3.1. Involvement of AT_1 but not AT_2 receptor in mediating AII response

The primary site of action of AII was examined using specific antagonist for AT_1 or AT_2 receptors: losartan or PD123177, respectively. As shown in Fig. 1, PD123177 (1 μM) did not have any significant effect on the AII-induced I_{sc}, 1.8 ± 0.4 μA/cm^2 ($n = 4$, $P > 0.05$, as compared to the control value of 1.5 ± 0.2 μA/cm^2). On the other hand, addition of losartan (1 μM) completely abolished the I_{sc} response to AII ($n = 3$), indicating the involvement of the AT_1 but not the AT_2 receptor in mediating the I_{sc} response to AII.

3.2. The involvement of Gi protein and PLC in mediating the Ca^{2+}-dependent I_{sc} response to AII

A previous study has demonstrated that AII elicited a transient increase in intracellular Ca^{2+} in CFPAC-1 cells [12]. To confirm that the AII-induced I_{sc} was Ca^{2+}-dependent, the effect of BAPTA-AM, an intracellular Ca^{2+} chelator, on the AII-induced I_{sc} was examined. It was found that treating the monolayers with BAPTA-AM (100 μM) for 45 min before the addition of AII (0.5 μM), the AII-activated I_{sc} was significantly reduced from 2.60 ± 0.13 to

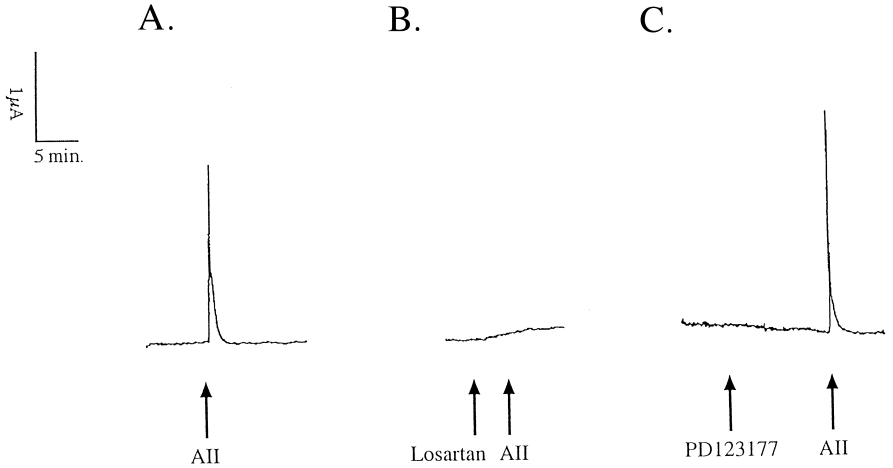

Fig. 1. Demonstration of the involvement of AII receptor type 1 (AT_1) in mediating the I_{sc} response. AII-induced (1 μM) I_{sc} recordings in control (A), losartan-treated (1 μM) (B), and PD123177-treated (1 μM) (C) epithelia. All drugs were added to the apical membrane. Note that AT_1 antagonist, losartan, but not AT_2 antagonist, PD123177, inhibited the AII-induced I_{sc} response.

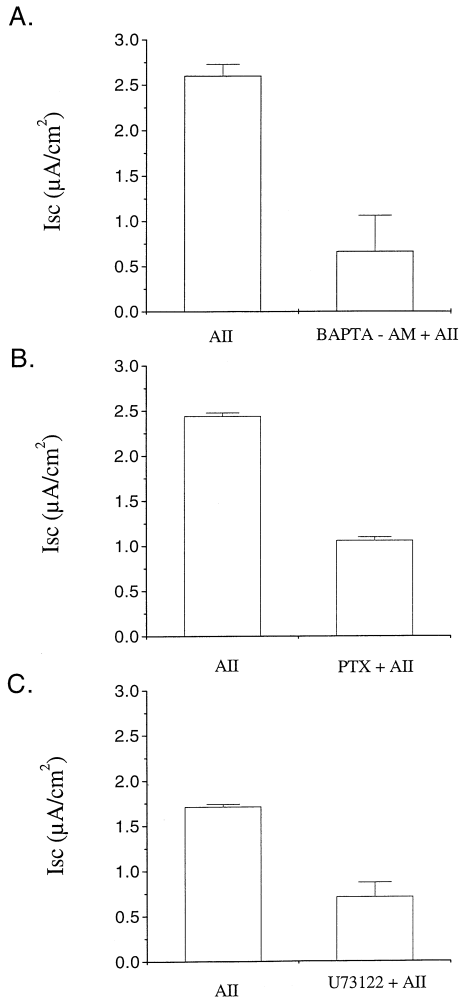

Fig. 2. Involvement of Gi and PLC in mediating the AII-induced Ca^{2+}-dependent I_{sc}. (A) Comparison of AII-induced I_{sc} responses in the absence and presence of a Ca^{2+}-chelator, BAPTA-AM (100 μM), treated 45 min prior to addition of AII. Inhibition of the AII-induced I_{sc} response by Gi inhibitor, pertussis toxin (100 ng, B) and PLC inhibitor, U73122 (5 μM, C). Cells were treated with pertussis toxin and U73122 for 24 h and 10 min, respectively, prior to addition of AII.

0.66 ± 0.40 μA/cm^2 ($n = 3$, $P < 0.01$), with a reduction of 74.6% (Fig. 2A).

The effect of a potent Gi protein inhibitor, PTX, on the AII-induced I_{sc} was also examined. PTX (100 ng/ml) was added to the monolayer 24 h before addition of AII (1 μM). The AII-induced I_{sc} was significantly reduced from 2.44 ± 0.08 to 1.06 ± 0.04 μA/cm^2 ($n = 12$, 56%, $P < 0.01$, Fig. 2B). U73122 (5 μM), a PLC inhibitor, added 10 min prior to the addition of AII (1 μM), induced a reduction of 58% in the AII-elicited I_{sc}, from 1.71 ± 0.03 to 0.71 ± 0.16 μA/cm^2 ($n = 4$, $P < 0.01$, Fig. 2C).

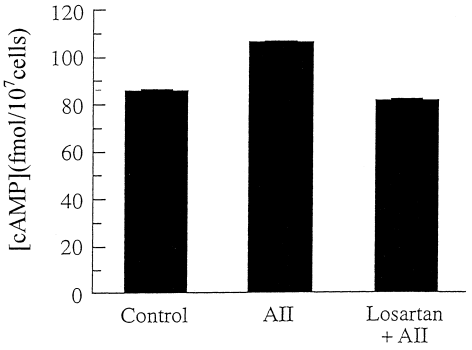

Fig. 3. AT_1 receptor-mediated increase in cAMP production. AII (100 μM), added to a 24-well plate for 10 min, induced a significant increase in cellular cAMP level ($P < 0.05$), which could be abolished by AT_1 antagonist, losartan (10 μM).

3.3. AII-induced cAMP production

It is controversial whether AII stimulates cAMP production in Cl^--secreting epithelia. The present study investigated the AII-stimulated cAMP production in CFPAC-1 cells by measuring intracellular cAMP level directly using RIA. As shown in Fig. 3, the cAMP content in AII-treated cells was significantly elevated as compared to the control ($P < 0.05$, $n = 4$). The AII-induced elevation of cAMP could be blocked by losartan, indicating the involvement of AT_1 receptor.

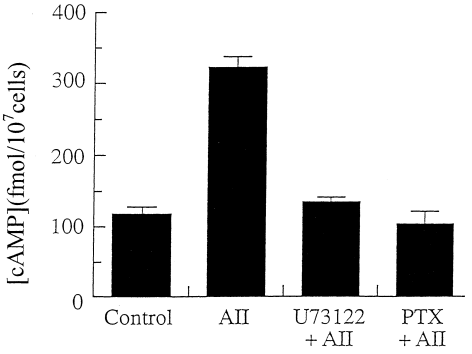

Fig. 4. Involvement of Gi and PLC in the AII-induced cAMP production. Inhibition of the AII-induced cAMP production by Gi inhibitor, pertussis toxin (100 ng/ml) and PLC inhibitor, U73122 (5 μM, $P < 0.02$). Cells were treated with pertussis toxin and U73122 for 24 h and 10 min, respectively, prior to addition of AII (100 μM).

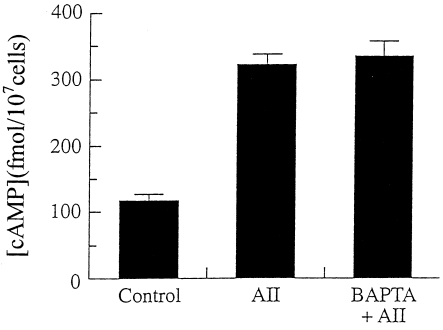

Fig. 5. Independence of the AII-induced cAMP production on intracellular Ca^{2+}. The cells were treated with BAPTA (100 μM) 45 min prior to addition of AII (100 μM).

3.4. Effect of PTX and U73122 on AII-induced cAMP production

The effect of PTX and U73122 on the AII-induced cAMP production was also examined. As shown in Fig. 4, the AII-induced cAMP production was significantly reduced ($P < 0.02$) when cells were treated with PTX (100 ng/ml, $n = 4$) and U73122 (5 μM, $n = 5$), 24 h and 10 min, respectively, prior to the addition of AII (1 μM), indicating a role of Gi and PLC in the AII-induced cAMP production.

3.5. AII-induced cAMP production was independent of intracellular Ca^{2+} and prostaglandin formation

The cAMP content was also measured in cells treated with 100 μM BAPTA-AM 45 min before addition of AII. It was found that BAPTA did not affect the AII-stimulated cAMP production ($n = 4$, Fig. 5). It was also found that pretreatment of the cells with cyclooxygenase inhibitors indomethacin (10 μM, Fig. 6) and piroxicam (10 μM, not shown) prior to AII stimulation did not have significant effect on the AII-induced cAMP production, excluding the involvement of prostaglandin formation.

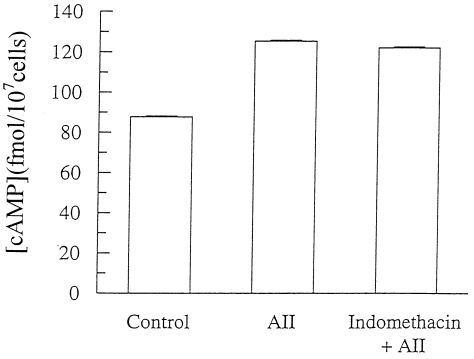

Fig. 6. Independence of the AII-induced cAMP production on prostaglandin formation. The cells were treated with indomethacin (10 μM) 10 min prior to addition of AII (100 μM).

4. Discussion

Separate signal transduction pathways involved in mediating the action of AII has been investigated using CFPAC-1 cell line which lacks cAMP-dependent Cl^- secretion but not cAMP production [19]. Therefore, AII-stimulated cAMP- and Ca^{2+}-dependent pathways can be studied separately using different techniques. Since the cAMP-dependent secretory pathway is defective in CFPAC-1 cells, the AII-induced I_{sc} response is likely to involve Ca^{2+} mobilization as indicated by the reduction in the AII-induced I_{sc} by the treatment with BAPTA to chelate intracellular Ca^{2+}. Although the AII-generated Ca^{2+} transient has also been suggested to be mediated by a Ca^{2+} influx through receptor-operated Ca^{2+} channel in adrenal glomerulosa [22] and proximal tubular epithelium [2,23], our previous study on CFPAC-1 cells has ruled out this possibility. The AII-induced I_{sc} was shown not to be dependent on extracellular Ca^{2+} since it was not affected when extracellular Ca^{2+} was chelated by EGTA [12]. The present study further suggests that the AII-induced Ca^{2+} mobilization involves pertussis toxin-sensitive PLC pathway as indicated by the observation that pertussis toxin and PLC inhibitor exerted an inhibitory effect on the AII-induced I_{sc}. This is consistent with the observation that PLC-induced increments in intracellular Ca^{2+} is the major signaling pathway coupled to AII receptors in many target tissues [3].

The signaling mechanism involved in mediating the AII-induced cAMP production has been controversial. While a decrease in cAMP production in response to AII, which is pertussis toxin-sensitive, has been observed in many organ tissues (for review see [2]), AII-induced secretory responses in a number of Cl^--secreting epithelia have been suggested to be due to an increase in cAMP production [8–11]. AII may stimulate cAMP production by activation of PLA_2

thereby releasing arachidonic acid from membrane phospholipids. Prostaglandins, which are converted from arachidonic acid by cyclooxygenase (COX), can then act on adenylate cyclase leading to elevation of intracellular cAMP [24,25]. Our direct measurement of cAMP using RIA on CFPAC-1 cells has confirmed an elevation of cAMP production in response to AII. However, COX inhibitors, indomethacin or piroxicam, were unable to block the AII-induced cAMP elevation, excluding the involvement of prostaglandin formation in mediating the AII-induced cAMP production. This is in contrast to what has been suggested in other epithelial cell types.

The fact that the AII-stimulated cAMP production in CFPAC-1 cells is sensitive to pertussis toxin and PLC inhibitor suggests that PLC-induced Ca^{2+} release may be responsible for the AII-stimulated cAMP production since elevation of intracellular Ca^{2+} has been shown to stimulate cAMP production in a number of cell types. Cytosolic Ca^{2+} has been recognized as one of the modulators of the cAMP cascade [26], through the interaction between calmodulin and adenylate cyclase [27]. Indirect activation of the cAMP-dependent regulatory pathway by extracellular ATP via an increase in $[Ca^{2+}]_i$ has also been observed in the rat epididymis [17,18]. However, the present study has ruled out this possibility since treatment of the cells with BAPTA did not have any significant effect on the AII-induced cAMP production. This result suggests that an alternative pathway may be involved in mediating the AII effect on cAMP production, detail mechanism of which awaits further investigation.

The important finding of the present study is that both the AII-induced Ca^{2+}-dependent secretory response and cAMP production are mediated by AT_1 receptors since AT_1 inhibitor, losartan, abolished both responses. Immunolocalization of AT_1 receptors has been previously demonstrated in CFPAC-1 cells [12]. However, it should be noted that the concentrations of AII used to elicit responses in the present study are higher than that found in human serum. This could be due to reduced expression of AT_1 receptors in the cell line as compared to that found in vivo. The AT_1 receptors on CFPAC-1 cells appear to be coupled to Gi and PLC to elicit further responses. This is consistent with the finding in most target tissues that PLC is the most dominant pathway coupled to AII receptors. What is interesting is that this pathway could lead to different responses, in the case of CFPAC-1 cells, Ca^{2+}-dependent secretory response and cAMP production. It is expected that the AII-induced cAMP production may elicit a cAMP-dependent secretory response in normal pancreatic duct cells since cAMP plays a major role in the regulation of pancreatic secretion [28]. AII receptor-coupled PLC pathway may also generate diacylglycerol release leading to activation of PKC [2]. AII-induced PKC activation has been shown to exert an inhibitory effect on the Ca^{2+}-dependent anion secretion in CFPAC-1 cells (Cheng et al., unpublished data). Taken together, AII may play a versatile role in modulating secretory responses in pancreatic duct cells through the action of pertussis toxin-sensitive PLC-coupled AT_1 receptors. However, the detailed signaling mechanism coupled to cAMP production in response to AII remains to be elucidated.

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